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1 **Full genome sequencing of porcine circovirus 3 fields strains from Denmark, Italy and Spain**
2 **demonstrates a high within-Europe genetic heterogeneity**

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4 Running title:

5 **PCV3 in Europe**

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18

19 **Summary**

20 *Porcine circovirus 3 (PCV3)* is a new species of the *Circovirus* genus, which has recently been
21 associated with different clinical syndromes. Its presence has been reported in different countries of
22 North and South America, Asia and recently also Europe (Poland). However, differently from the
23 other continents, no European PCV3 sequence is currently available in public databases. There is a
24 strong need of epidemiological data and full genome sequences from Europe because of its
25 relevance in the understanding of PCV3 molecular epidemiology and control. To fill this lack of
26 information, samples collected in Denmark, Italy and Spain in 2016 and 2017 were screened for
27 PCV3. Of the Danish samples, 36/38 of the lymph nodes, 6/20 serum samples and 2/20 lung

28 samples tested positive. Similarly, 10/29 lungs, 20/29 organ pools, 6/33 sera and 1/8 nasal swabs
29 tested PCV3 positive in Italy. Fourteen out of 94 serum pools from 7/14 Spanish farms were also
30 positive. Despite the convenience nature of the sampling prevents any precise prevalence
31 estimation, the preliminary screening of the data from three European countries confirmed a rather
32 wide PCV3 distribution in Europe. Furthermore, the analysis of the six obtained complete European
33 PCV3 genomes and their comparison with the public available sequences seems to support a
34 remarkable worldwide PCV3 circulation. These results underlines once more the urgency of more
35 extensive epidemiological studies to refine the current knowledge on PCV3 evolution, transmission,
36 spreading patterns and impact on pig health.

37 **Keywords**

38 Porcine circovirus 3, Europe, Full genome, Molecular epidemiology

39

40 **Introduction**

41 The *Circovirus* genus includes non-enveloped viruses with a single stranded circular genome of
42 approximatively 2kb. The tropism of these viruses was traditionally considered limited to a
43 restricted number of avian species and to swine (Todd, 2004). More recently, circoviruses have
44 been proven to infect several host species, belonging to different animal classes. Nevertheless, their
45 causative role in overt clinical disease is still unclear or marginal in most instances (Delwart and Li,
46 2012). The main exception is represented by the porcine circovirus 2 (PCV2), which has emerged
47 as one of the most widespread and devastating diseases affecting swine farming (Segalés, 2012;
48 Franzo et al., 2016). In 2016, a new circovirus species, named *Porcine circovirus 3* (PCV3) was
49 identified by deep sequencing in the USA (Palinski et al., 2017). Since then, several reports have
50 described its presence in China (Zheng et al., 2017), Poland (Stadejek et al., 2017) and Korea
51 (Kwon et al., 2017), supporting a worldwide distribution. Similarly to PCV2, PCV3 has been
52 associated with various clinical outcomes and lesions, including porcine dermatitis and nephropathy
53 syndrome (PDNS), reproductive disorders, respiratory signs (Palinski et al., 2017; Ku et al., 2017;
54 Shen et al., 2017) and myocarditis (Phan et al., 2016). Nevertheless, its presence in asymptomatic
55 animals has also been reported (Zheng et al., 2017) and definitive evidences of its virulence are still
56 lacking.

57 Being a single stranded DNA virus, PCV3 is expected to display a high evolutionary rate and
58 therefore the knowledge of its molecular epidemiology is of pivotal importance. In fact, the
59 availability of viral genome sequences represents a fundamental substratum for the understanding of
60 viral spreading patterns and for planning adequate control measures (Kühnert et al., 2011; Scotch et
61 al., 2011).

62 Thus, even though PCV3 has been reported in a single country in Europe (Stadejek et al., 2017), no
63 European PCV3 sequences are currently publicly available. To fill this gap, the present study
64 reports the first European PCV3 complete genome sequences, obtained by the joined efforts of three

laboratories located in Denmark, Italy and Spain, further supporting the wide distribution of this virus in Europe.

Materials and Methods.

Samples

A total of 271 samples were included in the study consisting of, 78 Danish (20 lungs, 20 serum and 38 lymph nodes), 99 Italian (29 lungs, 29 organ pools, 33 sera and 8 nasal swabs) and 94 Spanish samples (serum pools).

Italian samples were randomly selected archived samples delivered to the Veterinary Infectious Disease laboratory (Dept. Animal Medicine, Production and Health, Padua University, Italy) for routine diagnostic purposes between 2016 and 2017. Samples originated from sows and gilts (13 samples), nursery (45 samples) and growing and finishing (41 samples) pigs. The Danish samples (lymph nodes and placenta of sows, lungs from pigs (age unknown) and serum from pigs) were delivered for different diagnostic purposes, including the evaluation of decreased farrowing rates presence of respiratory disease and PCV2 viral load quantification. The Spanish samples were part of a longitudinal study in which 4-6 serum pools per farm were obtained from pigs at the end of the nursery and/or beginning of fattening periods (starting collection at 7 or 8 weeks of age and finishing it between 12 and 14 weeks of age). Each pool corresponded to 10 animals of the same age, collected longitudinally on a weekly basis, from a total of 15 farms. All studied farms were considered healthy (no evident clinical signs) and selection of pigs to be bled was performed randomly.

PCV3 diagnosis and sequencing

Italian and Spanish samples were extracted using the ExtractSpin TS kit (Bio-Cell, Rome, Italy) and tested for PCV3 using the real-time PCR described by Franzo et al., 2017 (Submitted).

Briefly, 2µL of extracted DNA were added to a standard mix composed by 1X DyNAmo Flash Probe qPCR Master mix, 0.6 µM and 0.3 µM of PCV3 specific primers and probe, respectively

91 (Table 1), 0.4 μ M and 0.2 μ M of internal control (IC) primers and probe (Hoffmann et al. 2006),
92 respectively and 5 pg of IC plasmid. Sterile nanopure water was added to bring the final volume up
93 to 10 μ L. The cycling parameters were 95° C for 7 min followed by 45 cycles of 95°C for 10 sec
94 and 60°C for 30 sec. The fluorescence signal was acquired at the end of each cycle extension phase.
95 Danish samples were extracted as described elsewhere (Hjulsager et al., 2009) and tested for PCV3
96 using the assay described by Palinski et al. (2017).

97 Full genome sequencing was attempted on all samples with a Cp lower than 30 (corresponding to a
98 viral titer of 100 copies/ μ L). Three primer pairs (Table 1) were used to amplify and sequence the
99 whole PCV3 genome by three overlapping amplicons. Two μ L of extracted DNA were added to a
100 standard mix composed of 1X Phusion®High-Fidelity mix, 200 μ M dNTPs, 0.6 μ M of each primer
101 and 0.5 units of Phusion DNA Polymerase. Sterile nanopure water was added to bring the final
102 volume up to 25 μ L. The following thermal protocol was selected: 98° C for 30 sec followed by 45
103 cycles of 98°C for 10 sec, 64°C for 20 sec and 72°C for 45 sec. A final extension phase of 5 min at
104 72°C was also performed. Amplification and specificity of bands were visualized using a SYBR
105 safer stained 2% agarose gel. DNA sequencing was performed at Macrogen (Macrogen Europe,
106 Amsterdam, Netherlands).

107 *Data analysis*

108 All chromatograms were visually inspected with Finch TV program. 1.4.0 (2004–2006 Geospiza
109 Inc) and consensus were obtained using the CromasPro (CromasPro Version 1.5; Technelysium Pty
110 Ltd, South Brisbane, Australia; <http://technelysium.com.au/wp/chromaspro/>). The sequences
111 were aligned with all PCV3 complete genomes available in GenBank (accessed 25/08/2017) using
112 MAFFT (Standley, 2013), and tested for recombination using GARD (Kosakovsky Pond et al.,
113 2006). Finally, a phylogenetic tree was reconstructed using the Maximum likelihood method
114 implemented in PhyML (Guindon et al., 2010) selecting as substitution model the one with the
115 lowest AIC calculated using JmodelTest (Posada, 2008). The robustness of the clade reliability was
116 evaluated by performing 1000 bootstrap replicates.

117 The raw genetic distance among strain pairs was calculated using MEGA6 software (Tamura et al.,
118 2013).

119

120 **Results and discussion**

121 Of the Danish samples, 36 out of 38 of the lymph nodes collected from sows were PCV3 positive,
122 as well as 6 out of 20 serum samples and 2 out of 20 lungs. Similarly, 10 out of 29 lungs, 20 out of
123 29 organ pools, 6 out of 33 sera and 1 out of 8 nasal swabs tested PCV3 positive in Italy. Fourteen
124 out of 94 (15%) serum pools from 7 (50%) out of 14 tested Spanish farms were also positive
125 (ranging from 1 to 4 positive pools, depending on the farm). Despite the convenience nature of the
126 sampling prevented any precise prevalence estimation, the results confirmed a rather wide PCV3
127 circulation in Europe, since it was initially detected in Poland (Stadejek et al., 2017). The virus was
128 detected in several tissues as well as in placenta-associated lymph nodes, supporting the broad and
129 systemic organ tropism of PCV3 (Palinski et al., 2017).

130 All six complete genome sequences (Acc.Numbers MF805719-MF805724) were 2000 nt long and
131 displayed two ORFs coding for 296 (*Rep*) and 214 aa (*Cap*) proteins, as previously described
132 (Palinski et al., 2017).

133 The sequences displayed p-distance distances from the USA isolates (Acc.Number KT869077)
134 ranging from 0.007 to 0.01. The Italian clade (mean within-clade genetic distance = 0.001) was
135 more closely related to strains collected in South Korea and Brazil (p-distance = 0.003)
136 (KY996341, KY996343, KY996341 and MF079254) while the Spanish sample demonstrated a
137 higher similarity with South Korean strains (p-distance = 0.003) (KY996341 and KY996343). The
138 two Danish sequences (mean within-clade genetic distance = 0.004) revealed the closest
139 relationship with strains detected in South Korea and China (p-distance = 0.006) (KY996338,
140 KY996341, KY996338 and KY075986). A heat Map reporting the p-distance calculated between
141 different sequence pairs of the analyzed sequences is displayed in Figure 1.

142 The phylogenetic tree based on the complete genome alignments demonstrated a tendency of the
143 sequences obtained in the present study to cluster according to the country of sampling.
144 Nevertheless, a more comprehensive analysis of the phylogenetic tree demonstrated a quite
145 different scenario characterized by a broad mixing of strains collected in different countries and
146 even continents. Particularly, even if the definition of a genetic cut-off is challenging and probably
147 misleading (Franzo et al., 2014), at least two groups can be potentially defined (Figure 2), both
148 including strains collected in North and South America, Asia and Europe. Remarkably, while
149 Danish sequences form a quite independent clade part of the B group (Figure 2), Italian and Spanish
150 ones were part of the A Group. Import of living pigs to Denmark is almost non-existing whereas a
151 large number of sows have been exported to other countries, including Korea and China. This may
152 explain the different grouping of the Danish sequences.

153 Based on these results, a single PCV3 introduction event is an unlikely justification for the
154 European PCV3 heterogeneity and for the phylogenetic relationship herein described. As already
155 described for PCV2 (Franzo et al., 2015), a worldwide PCV3 circulation leading to multiple
156 introduction events in different European countries followed by independent local evolution appears
157 a more likely scenario. . This is further supported by the demonstration of the PCV3 infection in
158 asymptomatic animals, which, together with the recent PCV3 identification, could have favored an
159 undetected and uncontrolled viral circulation.

160 Unfortunately, the paucity of currently available information hampers any definitive statement and
161 further studies and more data will be necessary to clarify PCV3 molecular epidemiology, its origin,
162 its impact on pig health and its transmission in and between countries and continents.

163 **Conflict of interest statement**

164 All authors have declared no conflict of interest.

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Primers/probes	Oligonucleotides	Assay
PCV3_353_F	5'-TGACGGAGACGTCGGGAAAT-3'	qPCR
PCV3_465_R	5'-CGGTTTACCCAACCCCATCA-3'	
PCV3_418_probe	5'-FAM-GGGCGGGGTTTGCGTGATTT-BHQ1-3'	
PCV3_74_F	5'-CACCGTGTGAGTGGATATAC-3'	PCR1 (Palinski et al.,2017)
PCV3_927_R	5'-CAAACCCACCCTTAACAG-3'	
PCV3_1303_F	5'-ACCGGAGGGGTCAGATTTAT-3'	PCR2
PCV3_541_R	5'-GAGCTGCTGCTTGAAGATCC-3'	
PCV3_817_F	5'-GTTATAATGGGGAGGGTGCT-3'	PCR3
PCV3_1647_R	5'-GCCTGGACCACAAACACT-3'	

Table 1

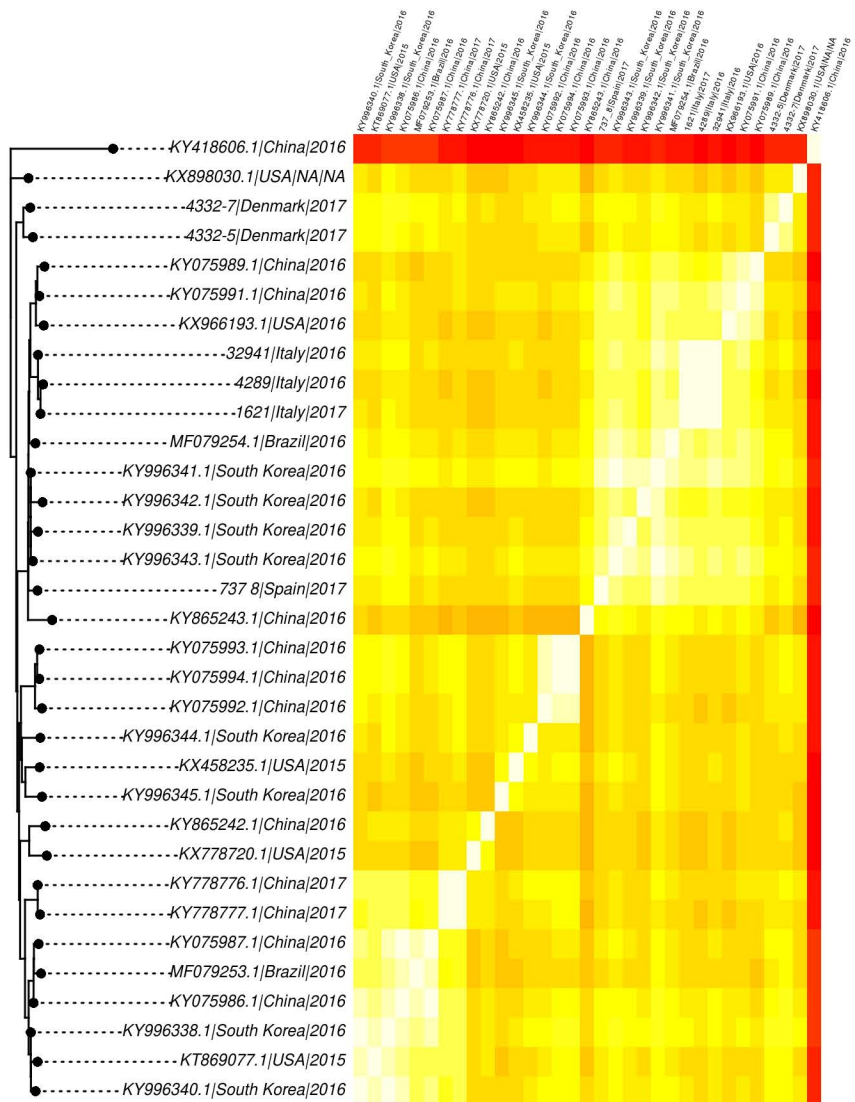
Primers and probes implemented in the assays described in this study.

Captions

Figure 1) Heat Map reporting the p-distance calculated between different sequence pairs. The relationship among strains is displayed through the maximum likelihood phylogenetic tree based on the full PCV3 genome.

Figure 2) Maximum likelihood phylogenetic tree based on the full PCV3 genome. The branch support is displayed in grayscale with darker black indicating higher bootstrap values. Danish, Italian and Spanish sequences are highlighted in blue, red and yellow, respectively.

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0 value 0.026

